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Tissue-specific regulation of 3'-nucleotide hydrolysis and nucleolar architecture

Benjamin H. Hudson and **John D. York**
Department of Biochemistry, Vanderbilt University Medical Center, 607 Light Hall, Nashville, TN 37232-0146, USA

Abstract

Sulfur is an essential micronutrient involved in diverse cellular functions ranging from the control of intracellular redox states to electron transport. Eukaryotes incorporate sulfur by metabolizing inorganic sulfate into the universal sulfur donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Sulfotransferases then catalyze the donation of the activated sulfur from PAPS to a broad range of acceptors including xenobiotic small molecules and extracellular proteoglycans while also generating the byproduct 3'-phosphoadenosine 5'-phosphate (PAP). In mammals, PAP is regulated by two related 3'-nucleotidases, Golgi-resident PAP phosphatase (gPAPP) and cytoplasmic bisphosphate 3'-nucleotidase 1 (Bpnt1), which hydrolyze PAP to 5'-AMP and whose inactivation results in severe physiological defects. Loss of Bpnt1 in mice leads to the accumulation of PAP in the liver, aberrant nucleolar architecture, and liver failure, all of which can be rescued by genetically repressing PAPS synthesis. Yet interestingly, Bpnt1 protein is expressed at high levels in a majority of tissues, suggesting that additional tissues might also be affected. To investigate this possibility, we closely examined the expression of Bpnt1 protein, accumulation of PAP, and appearance of dysmorphic nucleoli in wild-type and Bpnt1$^{-/-}$ mice. Surprisingly, we found that while Bpnt1 protein is widely expressed, only the liver, duodenum, and kidneys contain high levels of PAP and nucleolar reorganization. We hypothesize that these tissues share commonalities such as being highly polarized and situated at the interfaces of fluid reservoirs that might enhance their susceptibility to loss of Bpnt1. These studies highlight the importance of PAP metabolism in extrahepatic tissues and provide a framework for future investigations into the function of Bpnt1 in the kidney and small intestine.

I. Introduction

The incorporation of sulfur into macromolecules and small metabolites is a universal component of life (Hudson and York 2012). In metazoans, sulfur is found in numerous capacities throughout the cell including the sulfur-containing amino acids methionine and cysteine, reduction/oxidation switches like glutathione, and extracellular proteoglycans such as chondroitin sulfate (Masselot and De Robichon-Szulmajster 1975; Masselot and Surdin-Kerjan 1977; Cortes et al. 2009; Takahashi et al. 2011). In order to generate these diverse molecules, cells must first transform the biologically unavailable precursor, inorganic sulfate, into the universal sulfur donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS), a process which is mediated by the action of the bifunctional enzymes PAPS Synthases 1 and 2 (Hudson and York 2012). Following the generation of PAPS, members of the

sulfotransferase superfamily (SULTs) coordinate the transfer of the activated sulfur to a diverse set of acceptor molecules while also generating the reaction byproduct 3'-phosphoadenosine 5'-phosphate (PAP) (Gamage et al. 2006; Cheng and Klaassen 2009). Finally, PAP is hydrolyzed to 5'-adenosine monophosphate (5'-AMP) by the related Golgi and cytoplasmic 3'-nucleotidases, gPAPP and Bpnt1 (López-Coronado et al. 1999; Spiegelberg et al. 1999; Frederick et al. 2008).

Recent work from our lab has demonstrated important roles for gPAPP and Bpnt1 in normal mammalian development and physiology (Frederick et al. 2008; Hudson et al. 2013). Inactivation of gPAPP or Bpnt1 in mice results in a broad array of non-overlapping physiological defects. More specifically, gPAPP deficient mice succumb neonatally to pulmonary insufficiency and display stunted bone growth as a result of impaired glycosaminoglycan sulfation, while mice lacking Bpnt1 develop severe liver pathologies that frequently result in liver failure and death (Frederick et al. 2008; Hudson et al. 2013). Importantly, we were able to directly assess the role of PAP in these mice by analyzing double mutants harboring both hypomorphic mutations in PAPS Synthase 2 and inactive alleles of either gPAPP or Bpnt1. Unexpectedly, we found that suppressing PAPS synthesis exacerbated the phenotypes of gPAPP null mice yet rescued the liver failure of Bpnt1 knockouts (Hudson et al. 2013). Thus, while the two proteins provide the same enzymatic activity, their inactivation, and the subsequent loss of PAP hydrolysis in distinct subcellular compartments, gives rise to completely different physiological consequences.

Of the two 3'-nucleotidases, Golgi-localized gPAPP evolved more recently and is found only in metazoans, while cytosolic Bpnt1 is conserved from bacteria to humans (Neuwald et al. 1992; Gläser et al. 1993; Murguía et al. 1995; Peng and Verma 1995; Quintero et al. 1996). Yet despite Bpnt1's conservation, bacteria, fungi, plants, and animals have evolved widely divergent uses for the sulfur donor PAPS, highlighting the universal necessity for Bpnt1 and cytoplasmic PAP hydrolysis (Patron et al. 2008; Hudson et al. 2013). In addition, our previous studies demonstrate that Bpnt1 is expressed in a variety of mouse tissues and that its inactivation results in defects in secreted protein production, nucleolar morphology, and ribosome biogenesis, pathways not directly linked to its previously ascribed function as a component of the liver-specific sulfation detoxification machinery. In order to provide a deeper understanding of the physiological role of Bpnt1 in mammals, we performed a thorough examination of its expression and function in a number of mouse tissues. Surprisingly, we find that despite its expression in nearly all tissues examined, the loss of Bpnt1 results in significant accumulation of its substrate PAP and appearance of aberrant nucleolar morphology only in liver hepatocytes, renal proximal tubule epithelia, and small intestine enterocytes. These tissues and cell types share a number of commonalities that help to shed light on Bpnt1's physiological role including being highly polarized cells that reside at fluid interfaces and their energy-intensive transport of large amounts of metabolites across their apical and basolateral membranes. Together, our data suggest that Bpnt1 is involved in more than just liver-specific sulfation detoxification and provide the underpinnings for additional studies aimed at deciphering these unique tissue-specific roles.

II. Results

Bpnt1 is expressed in a majority of tissues

In order to investigate the physiological role of Bpnt1 in mammals, we first sought to understand its relative expression in a variety of mouse tissues (Figure 1) (Hudson 2013). Using immunoblotting, we found that Bpnt1 is expressed at relatively similar levels in a majority of tissues including the brain, heart, lungs, spleen, pancreas, stomach, liver, small intestine, colon, kidneys, and testes. Closer inspection by immunohistochemistry revealed that Bpnt1 is most enriched in the enterocytes of the small intestine and the proximal tubule

and thick ascending limb epithelia of the kidneys (data not shown). Other tissues including the brain, pancreas, stomach, liver, colon, and testes all expressed slightly lower, but comparable levels of Bpnt1. This broad expression along with the specific cellular enrichment suggest that Bpnt1 may have both common and tissue-specific functions.

Loss of Bpnt1 results in tissue-specific accumulations of PAP

To provide more insights into Bpnt1's potential tissue-specific functionality, we examined the levels of Bpnt1's substrate PAP in the same cohort of tissues as we did for the protein expression. Our previous studies demonstrated that the accumulation of PAP is directly responsible for the observed liver failure of Bpnt1 null mice. Therefore, we hypothesized that tissues with the highest levels of Bpnt1 protein would closely correlate with the quantity of PAP accumulation. Surprisingly, while we detected greater quantities of PAP in all Bpnt1 knockout tissues relative to wild-type, we found that the liver accumulated 3 to 4-fold more PAP (~300 nmole/g) than the next closest tissue, the kidney (~80 nmole/g). Proximal small intestine (duodenum), stomach, and heart also accumulated significant amounts of PAP (Fig. 2). That the liver accumulated the highest levels of PAP despite its moderate protein expression demonstrates that Bpnt1 protein levels do not correlate directly with the accumulation of its substrate and that additional mechanisms are responsible for determining how tissues are affected by the loss of Bpnt1.

Loss of Bpnt1 results in tissue-specific alterations to nucleolar morphology

The disconnect between accumulated PAP and Bpnt1 protein levels prompted us to search for additional markers of tissues that are negatively affected by the loss of Bpnt1. We had observed previously that Bpnt1 deficient hepatocytes undergo significant morphological rearrangements to their nuclei and nucleoli that correlated with the severity of liver disease. In the absence of Bpnt1, hepatocyte nuclei appear significantly hypertrophied with a prominent reduction in membrane-localized heterochromatin and a single large condensed nucleolus. We looked for the presence of these aberrant nuclei and nucleoli in multiple tissues by immunostaining for the nucleolar-resident protein fibrillarin and by electron microscopy. In addition to hepatocytes, we detected condensed nucleoli in two additional tissues, enterocytes of the early small intestine and proximal tubule epithelial cells of the kidney (Fig. 3). We were unable to detect aberrant nuclei in the brain, heart, lungs, stomach, colon, or pancreas, although we cannot discard the possibility that select cellular populations in these tissues are affected and remain overlooked. Thus, the appearance of aberrant nuclei and nucleoli correlated best with the PAP accumulation and not the protein levels.

III. Discussion

What properties do hepatocytes, proximal tubule epithelia, and enterocytes have in common that might provide insights into the physiological role of Bpnt1? One possibility is that all three cells mediate the absorption, movement, and transport of large volumes of solutes and solvents . Hepatocytes, the principle metabolic cell of the liver, provide numerous physiological functions including absorbing and filtering waste products from the blood. Similarly, enterocytes of the small intestine act at the interface of the digestive tract and the blood stream, transporting large quantities of water and nutrients from the digested food, while the proximal tubule performs a similar role in reabsorbing crucial salts and metabolites from the urine after it passes through the glomerular filter. These shared characteristics suggest that Bpnt1 may be involved in clearing the potentially toxic intracellular accumulation of PAP following its absorption from the blood, digestive tract, or urine filtrate.

One question resulting from these studies is why the liver accumulates 3-4 fold more PAP than the kidneys or duodenum despite expressing lower levels of Bpnt1 protein. The first potential explanation may be the relative flux of sulfur metabolites in the respective tissues and cell types. As discussed above, the liver provides the principle mechanism for the detoxification of endogenous and xenobiotic metabolites from the blood. One component of this process is the sulfation of small molecules, which universally requires PAPS, the precursor of PAP, to provide the reactive sulfate group (Klaassen and Boles 1997; Alnouti and Klaassen 2006). In order to generate the necessary PAPS, inorganic sulfate is brought into the cell and conjugated to adenosine via the activity of PAPS Synthases 1 and 2 (Hudson and York 2012). While present in other tissues, the sulfation detoxification pathway operates predominantly in the liver. Thus, hepatocytes likely experience greater rates of sulfate import, metabolism, and turnover than other cell types, which may contribute to the greater accumulation of PAP in the liver relative to other tissues.

A second possibility to explain the discrepancy in PAP accumulation is the relative proportion of affected cells in each tissue. The liver is composed chiefly of hepatocytes, which comprise roughly 70% of its total mass. In contrast, the kidney is a more complex tissue with greater compartmentalization and cellular diversity with the proximal tubule epithelia representing only a small portion of the whole tissue. This suggests that while proximal tubule epithelial cells may accumulate more PAP than hepatocytes on a per cell basis, the additional renal cells dilute this effect when examining the tissue as a whole. However, this hypothesis fails to explain why the liver expresses lower levels of Bpnt1 protein than the kidney and also why the small intestine, in which enterocytes comprise a significant percentage of total cells, does not accumulate even higher levels of PAP.

Finally, it is possible that different tissues use Bpnt1 to hydrolyze distinct substrates. For example, while PAP may predominate in the liver because of the abundant sulfation detoxification machinery, additional as-yet-undescribed 3’-phosphorylated nucleotides may be more abundant in the kidney and small intestine. Further work will be necessary to delineate these various possibilities.

Together, our data support our previous conclusion that the accumulation of PAP is responsible for the physiological defects of Bpnt1 null mice and also that the appearance of a hypertrophied nucleus combined with a condensed nucleolus can provide a reliable biomarker for cells and tissues affected by loss of Bpnt1. This is significant in two ways: i) the aberrant nucleolar appearance is relatively undescribed in the literature, as we were only able to find similar findings in the context of stimulated ribosome biogenesis and following hepatotoxic thioacetamide administration (Reynier et al. 1975; Kim et al. 2000; Jeong et al. 2001), and ii) although we would predict that genetic mutations affecting BPNT1 activity in humans would result in severe physiological deficiencies as they do for gPAPP (Vissers et al. 2011), there have been no clinical reports implicating BPNT1 in human disease. Thus, characterization of the nucleolar biomarker presented here will likely assist in the diagnosis of as-yet-undescribed BPNT1 deficiency syndromes.

Methods and Materials

All methods and materials have been described previously including the generation of Bpnt1 deficient mice and Bpnt1 specific antibodies, the development of a quantitative PAP assay, and the analysis of nuclear and nucleolar morphology in tissues by immunostaining with anti-fibrillarin antibodies. TEM analysis of the kidney was performed by the Vanderbilt University Electron Microscopy Core.

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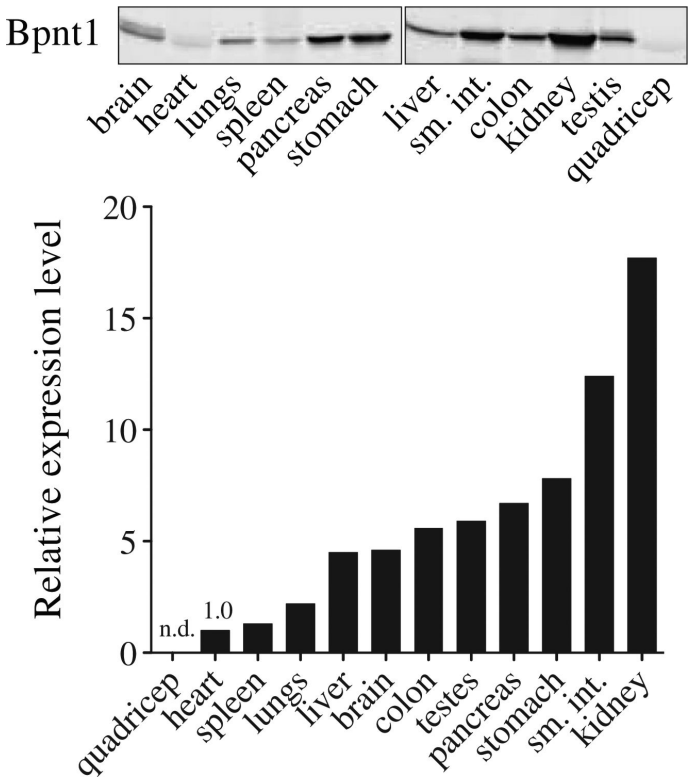

Figure 1.
Western blot of Bpnt1 expression in wild-type mouse tissues normalized to total soluble protein.

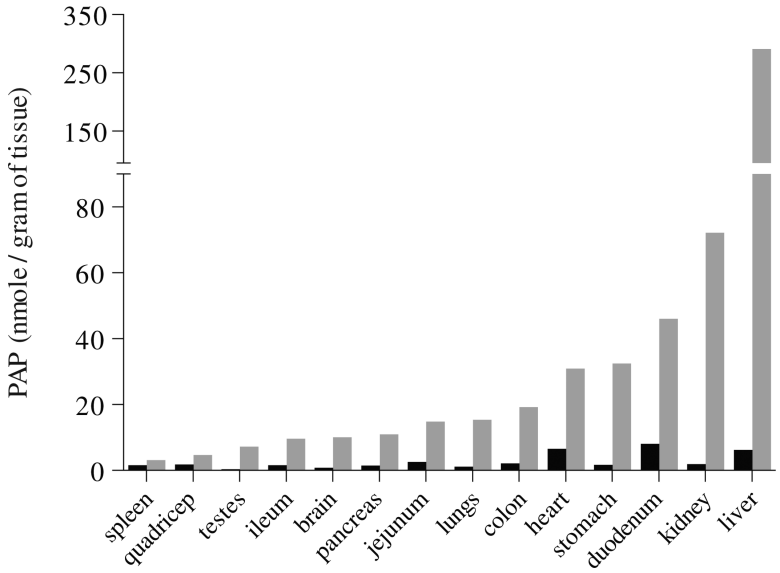

Figure 2.
PAP levels in wild-type and Bpnt1 knockout mouse tissues demonstrating significant accumulation in the liver, kidneys, and small intestine.

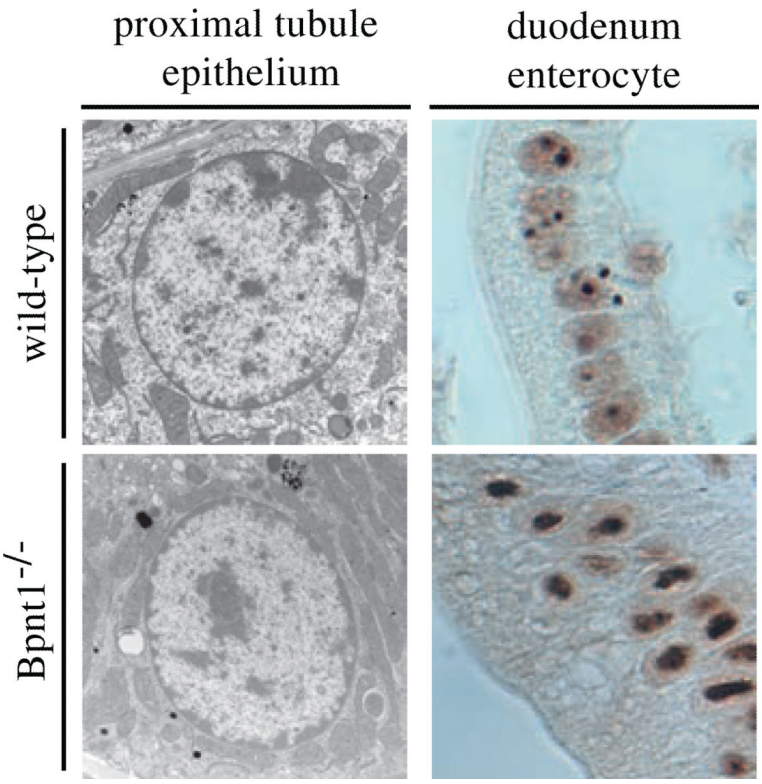

Figure 3.
Transmission electron micrograph and immunohistochemistry for the nucleolar-localized fibrillarin in renal proximal tubule epithelia and proximal small intestine enterocytes.